Molecular Epidemiology of Outbreaks of Viral Gastroenteritis in New York State, 1998–1999

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This investigation evaluated the role of Norwalk-like virus (NLV) and other viruses (rotavirus, enteric adenovirus, and enterovirus) in 11 outbreaks of acute nonbacterial gastroenteritis that occurred in multiple settings in a span of 18 months in New York State. To determine the etiology of illness, patients' stool specimens were analyzed with a combination of reverse-transcription polymerase chain reaction (RT-PCR) and nucleotide sequencing, cell culture, and ELISA diagnostic techniques. NLV was detected from all of these outbreaks, with an overall detection rate of 64% (51 of 79) for all specimens tested. Repeated attempts to isolate other viral pathogens were unsuccessful. Phylogenetic analysis of a subset of 27 specimens from these outbreaks showed the presence of both genogroup I and genogroup II NLVs. A spectrum of different nucleotide sequences were detected, demonstrating interoutbreak sequence variation and unrelated infections. NLV is a significant causative agent of diarrhea outbreaks in New York State.

Norwalk-like viruses (NLVs), which were previously called human calicivirus, the Norwalk family of viruses, or small round-structured virus (SRSV), are a genus of the family Caliciviridae [1]. The virus contains a single positive-stranded RNA genome 7700 nt in length and a single capsid protein of ~58 kDa in weight. Study of these viruses has been limited, because they can neither be grown in tissue culture nor propagated in an animal model. The determination of the complete genomic sequence of the prototype Norwalk virus [2, 3] and Southampton virus [4] and analysis of the immune responses of infected patients to recombinant virus protein antigens [5] have facilitated phylogenetic characterization of additional NLV strains [6]. As a consequence, these viruses can now be differentiated into 2 major genogroups: genogroup I, which contains Norwalk virus and Southampton virus, and genogroup II, which contains Hawaii virus, Camberwell virus, and Snow Mountain virus [7–11].

NLVs have been recognized as the most common viral cause of foodborne and waterborne outbreaks of acute gastroenteritis in the United States and elsewhere [1, 12–15]. The disease is transmitted via contaminated food or water, directly via person-to-person contact, and also by airborne droplets produced during vomiting. According to a recent estimate, ~23 million persons develop the disease each year in the United States [14].

Although the epidemiology of and risk factors for NLV disease are becoming more clear, little is known about the public health importance of NLVs as causes of epidemic and sporadic gastroenteritis among adults at a local or state level. This issue becomes even more important for localities that regularly host many large recreational activities (e.g., summer camps and skiing resorts) and/or house many nursing homes and similar

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senior-care facilities. Furthermore, New York State actively participates in the Centers for Disease Control and Prevention (CDC) Foodborne Disease Active Surveillance Network (FoodNet). An important objective of the program is to critically examine the role of NLVs in nonbacterial disease outbreaks. Thus, the increased awareness of NLVs as causes of epidemic gastroenteritis and the availability of better methods of detection of the virus led us to implement more-extensive viral testing (for calicivirus, rotavirus, enteric adenovirus, and enterovirus), using a combination of RT-PCR, cell culture, and ELISA techniques to determine the etiology of illnesses from outbreaks of gastroenteritis for which microbiologic tests had been negative for bacterial pathogens (which account for 66%–74% of gastroenteritis outbreaks) within New York State. Although FoodNet is active in selected New York counties, we looked at outbreaks statewide in order to have a larger database. So far, we have investigated NLV involvement in >45 outbreaks throughout the state that occurred from January 1998 through June 2001. In this report, we present data from 11 of these outbreaks, selected at random, that were investigated from January 1998 through July 1999.

METHODS

Outbreaks. Between January 1998 and July 1999, an attempt was made to collect stool specimens for viral analysis from gastroenteritis outbreaks in New York State that were relatively large in size (i.e., >10 cases) and for which microbiologic tests had been negative for bacterial pathogens. The study was conducted in accordance with guidelines for human research specified by the US Department of Health and Human Services.

Specimens. A representative sample of 79 (~78%) of 101 stool specimens from 11 (58%) of 19 outbreaks throughout the state were considered for this report. Three of the outbreaks occurred in counties (Rensselaer, Glendale, and Greene) that are FoodNet sureveillance areas (also known as "FoodNet sites"). The specimens, which were reported to be free of pathogenic bacteria by the local investigators, were kept frozen at −20°C until used.

Tissue culture. Conventional tube cultures of African green monkey kidney, human embryonic lung (HEL), human rhabdomyosarcoma (RD), and A549 cells were inoculated with a 10% suspension of each stool sample, which had been prepared in 0.05% gelatin-Tris Hanks' salt solution that contained 100 U/mL penicillin and 100 μg/mL streptomycin. The media used were prepared in-house by the Cell Culture and Media Preparation Section of the Wadsworth Center (New York State Health Department). In brief, confluent cultures were inoculated with 0.2 mL of stool suspension per tube and incubated at 37°C for 1 h. Next, the inoculum was removed and the cultures were incubated in maintenance medium (either Eagle's minimum essential medium [EMEM] with 25 mmol Hepes that contained the antibiotics, 50 U/mL fungizone, and 2% fetal bovine serum, for A549 and RD cells; or, for HEL cells or EMEM, 5% serum with 15% tryptose phosphate broth that contained the antibiotics and fungizone for the monkey cells). The inoculated cultures were checked frequently every (24–48 h) for cytopathic effects for 5–7 days.

ELISA. An ELISA kit (Pathfinder Rotavirus Kit 79673; Sanofi Diagnostics) was used for detecting rotavirus in the stool samples.

RNA extraction. Stool homogenates in sterile distilled water (concentration, 10%–20%) were mixed by vortexing with an equal volume of 1,1,2-trichloro-1,2,2-trifluoroethane (Freon) and clarified by centrifugation for 10 min at 1300 *g*. RNA from the aqueous supernatant was extracted by use of the Ultraspec-3 RNA Isotation System (Biotecx Laboratories).

RT-PCR primers. Two sets of primers were used. The set 1 primers, designated "DG" or "Region B" and supplied by the Viral Gastroenteritis Section of the Centers for Disease Control and Prevention (Atlanta, GA), were as follows: MON431 (5′-TGGACIAGRGGICCYAAYCA-3′), MON432 (5′-TGGACIC-GYGGICCYAAYCA-3′), MON433 (5′-GAAYCTCATCCAYCT-GAACAT-3′), and MON434 (5′-GAASCGCATCCARCGGAA-CAT-3′). MON433 and MON434 were used for reverse cDNA synthesis, and MON431 and MON432 were used for forward synthesis. The amplicon size was 213 bp. The set 2 primers, designated "51-3," were 51, with the sequence 5′-GTTGACAC-AATCTCATCATC-3′, and 3, with the sequence 5′-GCACCAT-CTGAGATGGATGT-3′ [16]. The amplicon size was 206 bp. These primers were designed to detect both genogroup I and genogroup II NLVs.

Conditions for RT-PCR. DG primer–dependent reverse transcription and PCR were carried out sequentially in a single reaction tube as described elsewhere [7]. In brief, RNA was suspended in buffer (50 μL) that contained 30 mmol of Tris-HCl (pH, 9.0), 100 mmol of KCl; 4.5 mmol $MgCl_2$; 0.2% (vol/vol) Triton-X; 1 mmol of dithiothreitol; 2 mmol of 2-mercaptoethanol; 40 U of human placental RNase inhibitor (Boehringer); 1.67 mmol each of 2′-deoxyadenosine–5′-triphosphate, 2′-deoxycytidine–5′-triphosphate, 2′-deoxyguanosine–5′-triphosphate, and 2′-deoxythymidine–5′-triphosphate; 1.2 μmol each of primers MON431, MON432, MON433, and MON434; 6 U of avian myeloblastosis virus super reverse-transcriptase (Molecular Genetic Resources); and 2.5 U of Ampli*Taq* DNA polymerase (Applied Biosystems). The thermocycle format of incubation was 1 cycle of RT at 42°C for 1 h, 1 denaturation cycle at 94°C for 3 min; 40 amplification cycles with denaturation at 94°C for 1 min, annealing at 46°C for 1 min 30 s, extension at 60°C for 2 min, and a final cycle of incubation at 72°C for 7 min. The amplification products were analyzed by electrophoresis in 3% agarose gel and visualized under UV

Table 1. Summary of 11 outbreaks of nonbacterial gastroenteritis for which stool specimens were tested for Norwalk–like virus (NLV) by RT-PCR, New York State, January 1998–July 1999.

County of outbreak (month and year)	Setting of outbreak	No. of sick persons	Mode of transmission	No. (%) of persons tested for NLV		
				Total	Male/female	With positive test result
Onondaga (Jan 1998)	Ski resort	1450	Waterborne	10	6/4	10 (100)
Rensselaer (Jun 1998)	School camp	81	Unknown	8	1/7	5 (63)
Warren (Dec 1998)	Nursing home	41	Person-to-person	14	4/10	5 (36)
Dutchess (Dec 98, Jul 99[b])	Delicatessen	32,150	Foodborne	4	1/3	4 (100)
Gowanda (Dec 1998)	Nursing home	187	Person-to-person	3	2/1	2 (67)
Glendale (Jan 1999)	Nursing home	165	Person-to-person	7	3/4	3 (43)
Sullivan (Jul 1999)	Summer camp	23	Person-to-person	10	3/7	5 (50)
Schuyler (Apr 1999)	Nursing home	NA	Person-to-person	5	1/4	4 (80)
Parker (Apr 1999)	Institution	NA	Person-to-person	7	3/4	4 (57)
Georgian (May 1999)	Restaurant	184	Foodborne[c]	7	3/4	6 (86)
Greene (Jul 1999)	Summer camp	106	Person-to-person	4	1/3	3 (75)
Total	…	2419	…	79	28/51	51 (64)

[a] Water mixed in beverage.
[b] Dec 98, "Duchess 1," 2 specimens; Jul 99: "Dutchess 2," 2 specimens.
[c] Improper food handling.

illumination after staining with 0.5 μg/mL ethidium bromide. The conditions for 51-3–dependent RT-PCR have been described elsewhere [16].

Nucleotide sequencing. The 213-bp or 206-bp product cDNA was excised from the gel, extracted and purified using Qiaex gel extraction kit (Qiagen), and subjected to automated nucleotide sequencing (ABI Prism 377; Perkin-Elmer Biosystems). Nucleotide and deduced amino acid sequences were analyzed by use of the University of Wisconsin Genetics Computer Group software package [17].

RESULTS

Eleven outbreaks of gastroenteritis (from which 79 specimens were available) occurred statewide in New York in a span of 18 months (January 1998–July 1999) (table 1). These outbreaks ranged in size from 23 to 1450 cases of illness (median, 168 cases). The outbreaks were scattered throughout the state and the time period, with no clear seasonal pattern. Various setting were involved: 4 outbreaks were associated with nursing homes, 3 with summer camps, 2 with eating establishments, 1 with a institution, and 1 with a ski resort. The suspected mode of transmission was person-to-person in 7 outbreaks, foodborne in 2, waterborne in 1, and unknown in 1. The specimens that were tested came from patients aged 11–97 years (median, 49 years); 65% of whom were female. The incubation period varied from 24 to 96 h (median, 60 h), and the duration of illness ranged from 10.5 to 57 h. Nausea was a prominent symptom, along with vomiting, diarrhea (nonbloody), fever, and chills; other symptoms—abdominal cramps and headaches—were less frequent (table 2).

The stool specimens tested were reported by local laboratories to be free of bacterial pathogens; the specimens tested negative for enteroviruses and enteric adenoviruses (serotypes 31, 40, and 41) by cell culture and for rotavirus by ELISA in our laboratory. However, 51 of the 79 of the specimens tested positive for NLV by RT-PCR (figure 1). Each primer set (DG and 51-3) produced a single major band of the expected size (213 and 206 bp, respectively); these were subsequently sequenced. NLVs were identified in all outbreaks, with a rate of detection of 64% (51 of 79 specimens) for all stool specimens tested. The number of specimens that tested positive for each outbreak varied from 36% to 100% (mean, 68%). Of the 51 positive specimens, 39 (76%) tested positive with the DG primers and 12 (23%) tested positive with the 51-3 set. Selected

Table 2. Disease symptoms experienced by the patients of nonbacterial gastroenteritis outbreaks.

Symptom(s)	No. (%) of patients
Nausea and/or vomiting	39 (49)
Diarrhea	50 (63)
Fever, chills	28 (35)
Abdominal cramps	6 (7.6)
Other (e.g., headache)	7 (8.8)

NOTE Thirty-eight patients (48%) experienced multiple symptoms.

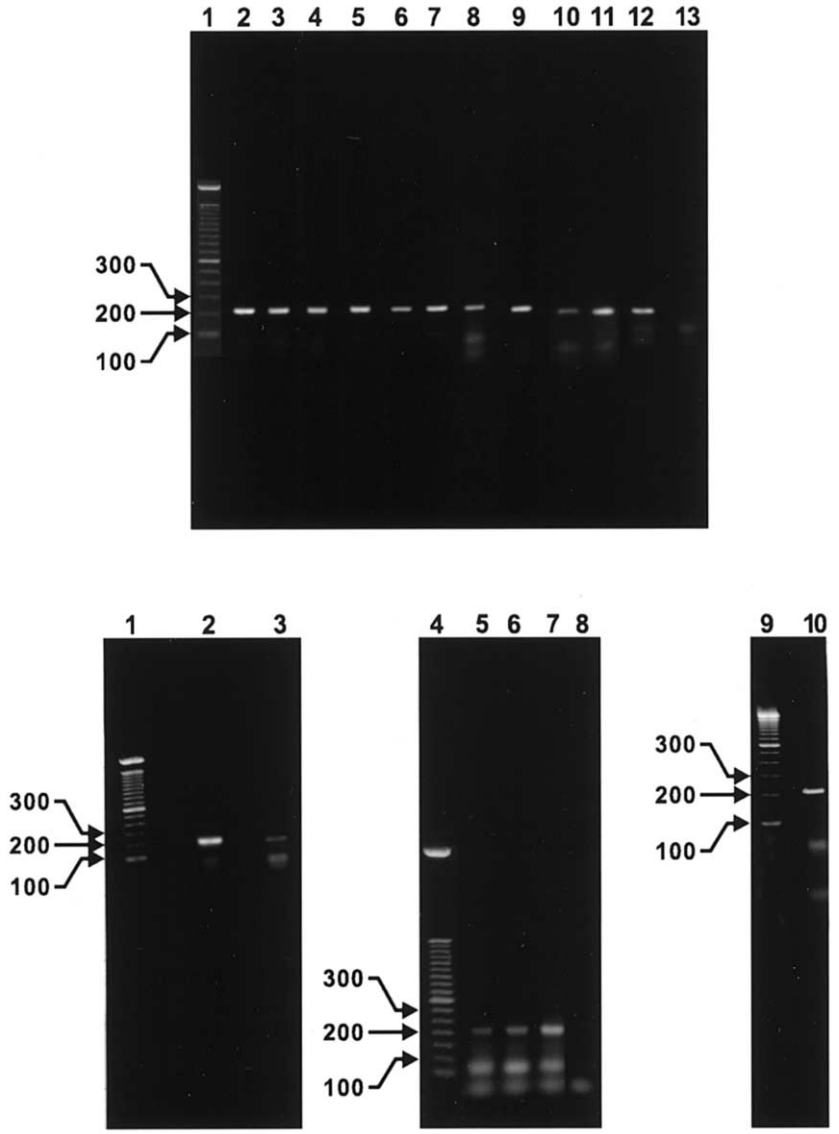

Figure 1. Genetic fingerprints of 213-bp *(upper panel)* and 206-bp *(lower panel)* RT-PCR products (in agarose gels after staining with ethidium bromide) of viral isolates from stool specimens from various gastroenteritis outbreaks. *Upper panel,* RT-PCR patterns obtained with use of DG primers for RNA from isolates from the following outbreaks: *lanes 2–4,* Schuyler; *lane 5,* Dutchess; *lanes 6–7,* Georgian; *lanes 8* and *9,* Dutchess; *lanes 10* and *11,* Parker; *lane 12,* Sullivan 1B; *lane 13,* water control; *lane 1,* 100-bp molecular marker. *Lower panel,* RT-PCR patterns obtained with use of 51-3 primers for RNA from isolates from the following outbreaks: *lane 2,* Glendale; *lane 3,* Warren; *lanes 5–7,* Rensselaer; *lane 10*, Onondaga; *lane 8,* water control: *lanes 1* and *9,* 100-bp molecular marker; *lane 4,* 50-bp marker.

DG-positive specimens were also analyzed for their cross-reactivity with the 51-3 primer pair. Of these, 10 specimens from 3 outbreaks (6 from the Onondaga, 1 from the Gowanda, and 3 from the Greene outbreaks) reacted with primers of both sets. These limited data demonstrate the higher detection efficiency of the DG set (76%), compared with the 51-3 set (23%), and indicate the great genetic diversity in the NLVs from these outbreaks.

To study this genetic variability further, we analyzed sequenced amplicons from 27 NLV isolates from these outbreaks. A 147-nt region toward the 3′ end of open reading frame (ORF) 1 [3, 16, 18] was obtained with the DG set of primers from 18 isolates from 7 outbreaks and compared with the corresponding sequences from reference strains. These strains included Desert Shield virus [19] (genetic cluster GI3) [6], Camberwell virus [8] (genetic cluster GII4), and the NLV strain Nor89JD (SRSV-OTH-25/89/J) [11] (genetic cluster GII3). The nucleotide sequence of each outbreak strain was unique and differed from that of the reference strains. Of the reference strains used, the Camberwell virus was most similar (up to 95% nucleotide sequence identity) to isolates from 4 outbreaks (the Georgian, Parker, Schuyler, and Sullivan outbreaks), the reference NLV

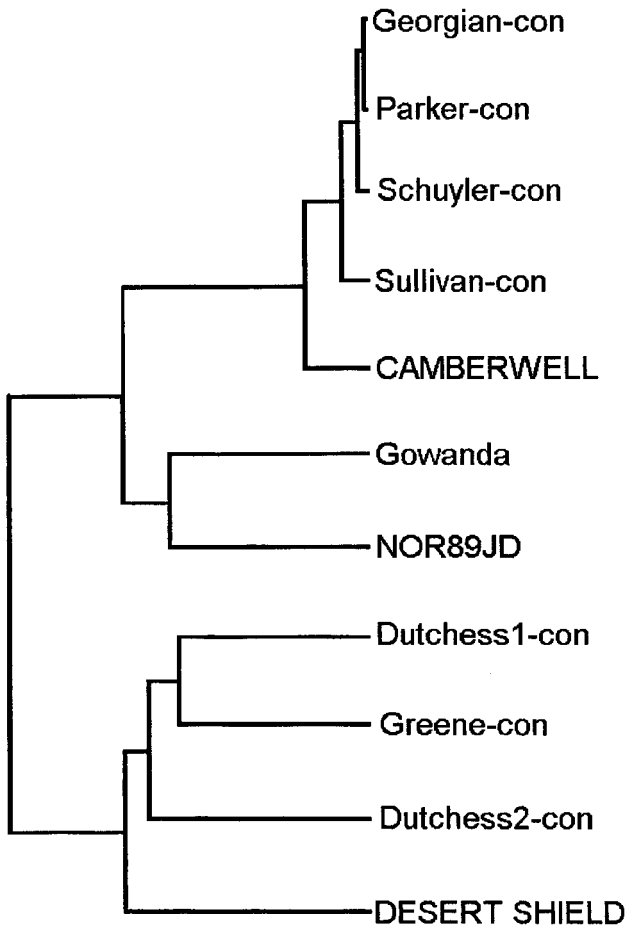

Figure 2. Dendrogram showing clustering relationship between calicivirus isolates from gastroenteritis outbreaks and reference strains Norwalk-like virus, based on nucleotide sequencing data. The distance along horizontal axis is proportional to nucleotide differences between sequences. The dendrogram was generated with the University of Wisconsin Genetics Computer Group software package [17]. Capital letters indicate reference strains; the suffix "con" indicates "consensus sequence."

strain Nor89JD was most similar to the isolate from the Gowanda outbreak (80% identity), and the Desert Shield virus was most similar to the isolates from the Dutchess 1, Dutchess 2, and Greene outbreaks (71%–75% identity) (figure 2). Isolates from the Dutchess 1 and Dutchess 2 outbreaks showed dissimilar sequences. The identity between outbreak isolates (i.e., interoutbreak identity) varied from 61% (Dutchess 2 outbreak strain vs. Georgian or Sullivan strains) to >90% (Georgian vs. Parker, Schuyler or Sullivan), whereas isolates from a single outbreak were similar to each other.

A 150-nt region of the viral RNA polymerase gene was obtained with the 51-3 primer pair from 9 other isolates from 4 different outbreaks and was compared with the corresponding sequences from the reference Norwalk virus [2, 3] and Southampton virus [4] (genetic cluster GI2) [6] (figure 3). We again observed that the sequence of each outbreak was unique and differed from those of the reference strains. The nucleotide sequence identity between the isolates and the reference virus ranged from 73% to 81%, and interoutbreak identity was >95% (Glendale outbreak strain vs. Onondaga or Warren strains).

Nucleotide substitutions occurred at the third nucleotide of codons in most of the instances we detected. The 49–amino-acid sequences deduced from the nucleotide sequences of the DG-amplified PCR products of the 18 isolates are shown in figure 4*A*. The isolates from 4 outbreaks (Georgian, Parker, Schuyler, and Sullivan) showed identical amino acid sequences and had a 100% sequence identity with the reference Camberwell virus; the isolate from Gowanda outbreak, with 6 amino acid substitutions, showed nearly 90% sequence identity, and the isolates from the remaining outbreaks (Dutchess 1, Dutchess 2, and Greene), each with several substitutions, showed lower sequence identities (66%–70%) to the reference virus. Amino acid substitutions were dissimilar in the isolates from the 2 outbreaks in Dutchess County. Thus, the isolates from these latter outbreaks (Gowanda, Dutchess 1, Dutchess 2, and Greene) showed interoutbreak diversity, as measured by the number of amino acid substitutions. Of the reference strains used, the Camberwell virus had a 84% amino acid identity with

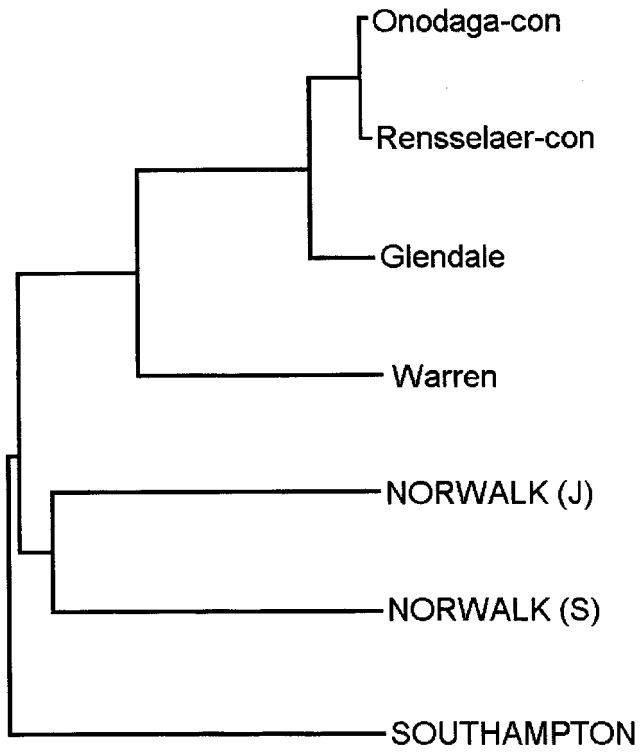

Figure 3. Dendrogram showing clustering relationship between calicivirus isolates from outbreak stool specimens and reference strains of Norwalk-like virus and Southampton virus, based on nucleotide sequencing data. The dendrogram was generated with the University of Wisconsin Genetics Computer Group software package [17]. Capital letters indicate reference strains; the suffix "con" indicates "consensus sequence."

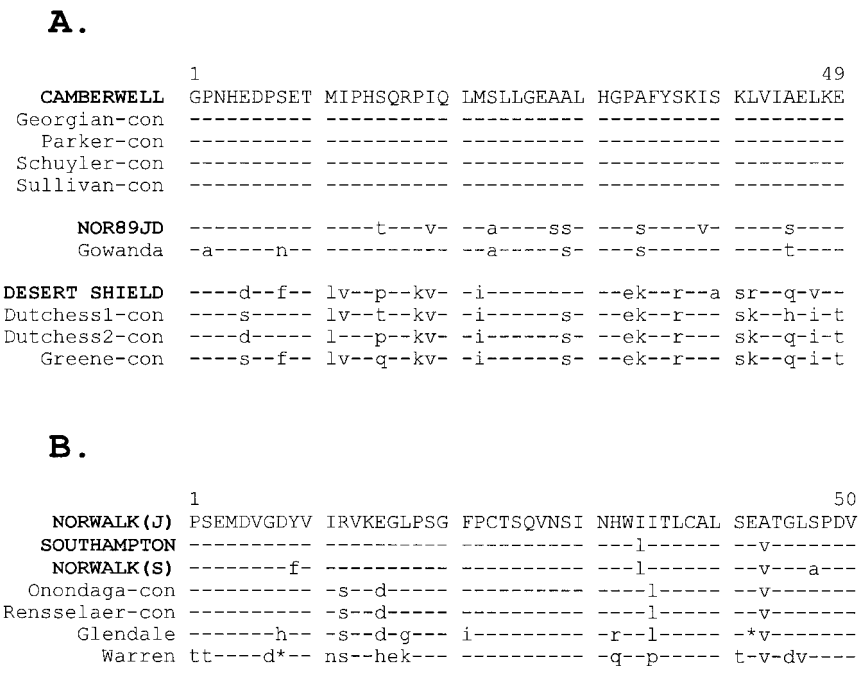

Figure 4. Deduced amino acid sequences of outbreak isolates and reference caliciviruses. Only amino acids that are different from the amino acids of reference Camberwell virus *(A)* and Norwalk virus *(B)* are shown. Hyphens denote identical amino acids.

Nor89JD strain but less identity (67%) with the Desert Shield virus.

The 50–amino-acid sequences of the remaining 9 isolates, as deduced from the nucleotide sequences of the PCR products obtained with the 51-3 primer pair, also showed some inter-outbreak diversity and diversity from the reference Norwalk virus. The number of amino acid substitutions ranged from 4, in the isolates from 2 outbreaks (Onondaga and Rensselaer), to several, in the isolates from 2 other outbreaks (figure 4*B*).

DISCUSSION

One of the objectives of the New York State Health Department and state epidemiologists has been to develop public health capacity to rapidly detect and investigate outbreaks of gastroenteritis by use of sophisticated epidemiologic and laboratory techniques. Such early detection will facilitate the implementation of control measures and the prevention of illness and death. Toward this end, the present study is one of the first statewide investigations (including 2 emerging infectious pathogens sites, Rochester and Albany) (figure 5) of a series of successive outbreaks of viral gastroenteritis (which occurred over a period of 18 months) in which a combination of RT-PCR, cell culture, and ELISA diagnostic methods were used to determine the etiology of illness by testing of bacteria-negative stool specimens. In the investigation, particular attention was paid to the detection of NLVs, enterovirus, rotavirus, and enteric adenovirus (serotypes 31, 40, and 41), which are the most commonly reported viral causes of gastroenteritis among adults and children [20]. The investigation also included an analysis of the genetic diversity of the NLVs collected from these chronologically separate occurrences, and the results uncovered several distinct patterns.

Of interest, NLVs were the sole viral agent identified by RT-PCR from all 11 nonbacterial outbreaks, with a detection rate of 64% (51 of 79 specimens) for all stool specimens tested. Attempts to identify other pathogens by culture and other tests in the outbreak specimens were repeatedly unsuccessful. In addition, the clinical features and symptoms of the illness, including the brief incubation period (24–96 h) and the duration of illness (10–57 h), and the high prevalence of nausea, vomiting, and diarrhea were consistent with the previously reported characteristics of illness associated with NLVs [13, 14, 20, 21]. The detection rate varied considerably between outbreaks, which could be due to differences in virus contamination of the specimens, the condition of the specimens (e.g., inhibitors), and the timing of their collection and/or storage prior to transfer to the testing site [20, 22, 23]. Nevertheless, our investigation confirms that NLVs are important pathogens causing outbreaks of diarrhea in New York State. Furthermore, in these outbreaks, the virus has been spread via contaminated food or water and directly from person to person, which is consistent with the modes of transmission reported in previous outbreaks of gastroenteritis [12, 20, 21]. Therefore, paying particular attention

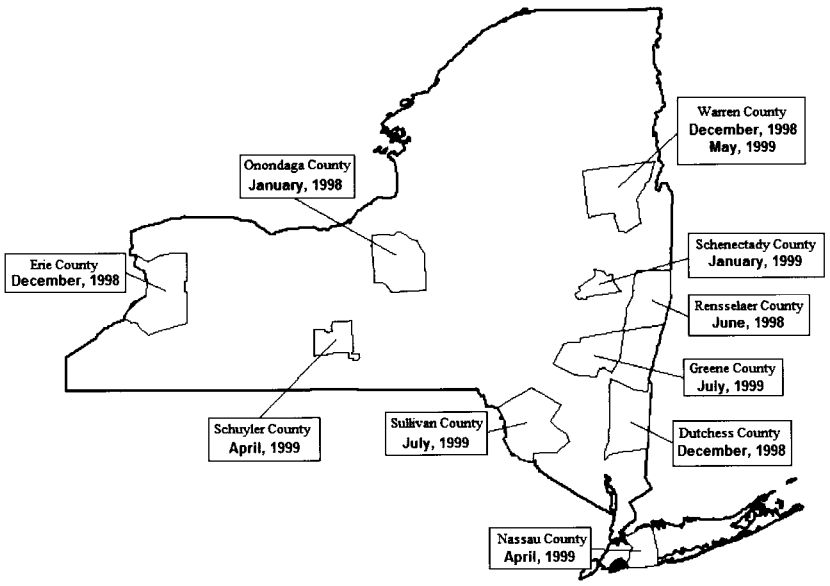

Figure 5. A map of New York State showing the counties where the sequential outbreaks of Norwalk-like virus gastroenteritis occurred in 1998–1999.

to the personal hygiene of food handlers and the adoption of a work policy that would include paid leave for employees with gastroenteritis, in various gatherings, would help reduce NLV transmission. The study can benefit all states (and the "100-Man Strong" study, another CDC project with a different scope) that are working to improve viral detection. For example, viral diagnosis kits and specimen mailers could be distributed to the sites that are in need of these items.

To track the molecular epidemiology of the infection, genetic fingerprint analysis of the RNA polymerase and 3′-terminus of the ORF1 region was performed for a subset of 27 RT-PCR–positive specimens from 11 outbreaks; these sequences were amplified with either the DG or the 51-3 primers. Our data demonstrated that there was considerable sequence variation among strains belonging to genogroups I and II and that genogroup II viruses were more common than those of genogroup I. Thus, a spectrum of different nucleotide sequences were detected in the viruses that we recovered, and none of the specimens contained sequences that were identical to those of the reference strains.

Interesting differences existed in the distribution of NLV genotypes recovered over time. The sequential outbreaks of April–May 1999—the Schuyler, Parker, and Georgian outbreaks—were caused by Camberwell-like viruses with great homology; there were only a few nucleotide changes in the RNA polymerase gene sequence between outbreak strains. However, later in the year, outbreak strains with a different pattern emerged; 3 genotypes clustering with Camberwell and Desert Shield viruses were detected in the Sullivan, Greene, and Dutchess 2 outbreaks. A Desert Shield virus–like genotype and a Nor89JD-like genotype, each with a few nucleotide changes, were also detected in the Gowanda and the Dutchess 1 outbreaks of December 1998. The 2 foodborne outbreaks in Dutchess county, which occurred within a span of ~7 months, showed different sequences, implying that there were separate introductions of the virus and that a single strain did not cause multiple outbreaks over time. On the other hand, the other outbreaks (Onondaga, Rensselaer, Warren, and Glendale) of January 1998 through January 1999 showed genotypes clustering with NLVs. Thus, this limited study shows that multiple virus genotypes caused the outbreaks and suggests that NLVs are an important public health problem for New York State residents of all ages.

Because most of the outbreaks proved to be viral in origin and, more specifically, proved to be caused by NLVs, RT-PCR and molecular sequencing need to be applied to link outbreaks with common strains, to trace outbreaks to vehicles of infection, and, ultimately, to improve public health practices, especially with respect to foodborne outbreaks and food handlers. Further clinical laboratory analysis and research with additional outbreak specimens and specimens from asymptomatic persons or from persons with diarrhea known to be caused by other pathogens are needed to gain better insight into the viral replication mechanisms and into food sources and to develop comprehensive preventive measures to reduce the incidence of viral gastroenteritis.

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